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Effects of local delivery of D-amino acids from biofilm-dispersive scaffolds on infection in contaminated rat segmental defects

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ABSTRACT

Infectious complications of open fractures continue to be a significant factor contributing to non-osseous union and extremity amputation. The persistence of bacteria within biofilms despite meticulous debridement and antibiotic therapy is believed to be a major cause of chronic infection. Considering the difficulties in treating biofilm-associated infections, the use of biofilm dispersal agents as a therapeutic strategy for the prevention of biofilm-associated infections has gained considerable interest. In this study, we investigated whether local delivery of D-Amino Acids (D-AAs), a biofilm dispersal agent, protects scaffolds from contamination and reduces microbial burden within contaminated rat segmental defects *in vivo*. *In vitro* testing on biofilms of clinical isolates of *Staphylococcus aureus* demonstrated that D-Met, D-Phe, D-Pro, and D-Trp were highly effective at dispersing and preventing biofilm formation individually, and the effect was enhanced for an equimolar mixture of D-AAs. Incorporation of D-AAs into polyurethane scaffolds as a mixture (1:1:1 D-Met:D-Pro:D-Trp) significantly reduced bacterial contamination on the scaffold surface *in vitro* and within bone when implanted into contaminated femoral segmental defects. Our results underscore the potential of local delivery of D-AAs for reducing bacterial contamination by targeting bacteria within biofilms, which may represent a treatment strategy for improving healing outcomes associated with open fractures.

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1. Introduction

Despite meticulous treatment, infectious complications of open bone fractures continue to be a significant factor contributing to patient morbidity and poor healing outcomes. The ensuing infection of bone by bacteria (osteomyelitis) is characterized by high levels of inflammation and destruction of viable bone tissue. Often the infection becomes chronic, resulting in increased rates of surgical revisions, non-union, and extremity amputation [1–3]. Among the pathogenic microorganisms associated with chronic osteomyelitis, *Staphylococcus aureus* is the most frequently isolated organism, accounting for >50% of all cases [4–8]. In addition to the increasing trend of antimicrobial resistance among clinical isolates, biofilm formation is a significant contributing factor in the development of both device and non-device related chronic orthopedic infections and a major barrier to wound healing [4,6].

Bacterial biofilms are an association of single or multiple species attached to a surface surrounded by an extracellular polymeric matrix (EPM), which constitutes a protected mode of growth. Compared to their planktonic counterparts, biofilm-derived bacteria have distinctive phenotypes in regards to growth, gene expression, and protein production that confer resistance to antimicrobial agents as well as host mechanisms of clearance [5,9]. Importantly, bacterial biofilms have been associated with a broad range of human infections, including chronic non-device-related infections such as osteomyelitis [10,11]. Previous studies have reported that staphylococcal biofilms are present within infected bone of patients with chronic osteomyelitis [6–8], and that clinical osteomyelitis isolates of *S. aureus* are capable of forming biofilms *in vitro* [12–14]. Furthermore, staphylococcal biofilms have been

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implicated as a major cause of osseous non-union [15]. These studies suggest that staphylococcal biofilms play a critical role in both the development of chronic osteomyelitis and the sequelae of infectious complications.

Biofilm development is a highly coordinated and reversible process beginning with the attachment and proliferation of cells on a surface and culminating in the dispersal of cells from the biofilm into the surrounding environment. The dispersal of cells is an essential stage of the biofilm life cycle that contributes to survival of the organism and disease transmission. For both Gram-positive and Gram-negative microorganisms, biofilm dispersion is mediated by self-produced diffusible factors [16,17]. Considering the specificity and effectiveness of these molecules for dispersing biofilms, the use of biofilm dispersal agents has attracted considerable interest for the treatment of biofilm-associated infections [18,19]. Recent studies have shown that use of biofilm dispersal agents, including bismuth thiols [20], recombinant DNAses [21], and diffusible soluble factors [22,23], can disperse biofilms *in vitro* and improve healing of biofilm-associated infections *in vivo* [24,25]. However, toxicity to viable host tissues (as observed for bismuth thiols and xylitol), as well as the specificity of these agents for certain bacterial species and/or strains, may preclude their use as broad therapeutic strategies.

Recent studies have shown that the D-isomers of amino acids (D-AAs) can prevent and disperse biofilms formed by a diverse range of bacterial species, including *S. aureus* and *Pseudomonas aeruginosa* [26,27]. In contrast to other biofilm dispersal agents, D-AAs promote the disassembly of biofilms through multiple mechanisms and have minimal cellular toxicity [28]. In this study, we investigated the ability of biofilm-dispersive polyurethane (PUR) scaffolds augmented with D-AAs to protect the scaffold from contamination from the contiguous wound environment and to reduce microbial burden within segmental defects *in vivo*. A mixture of D-AAs with optimal *in vitro* anti-biofilm activity was evaluated in a rat contaminated segmental defect model to test our hypothesis that local delivery of D-AAs will reduce the extent of infection within the defect. We also investigated the cytotoxicity of D-AAs on host mammalian cells to further evaluate their therapeutic potential.

2. Materials and methods

2.1. Materials

D- and L-isomers of amino acids (free base form), including alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine, and valine, were purchased from Sigma–Aldrich (St. Louis, MO, USA). For use in bacterial and cell cultures, D-AA stocks were prepared by dissolving powders in 0.5 M HCl at concentrations between 150 and 200 mM. Stocks were then diluted into cation-adjusted Mueller Hinton (MHB-II) broth neutralized to pH 7.4 and stored at −80 °C. For polyurethane scaffold synthesis, ε-caprolactone and stannous octoate were supplied by Sigma–Aldrich, and glycolide and D, L-lactide were purchased from Polysciences. An isocyanate-terminated prepolymer (22.7% NCO) comprising polyethylene glycol (PEG) end-capped with lysine triisocyanate (LTI) at a 2:1 M ratio of LTI:PEG was supplied by Medtronic (Memphis, TN, USA). Triethylene diamine was purchased from Evonik (TEGOAMIN 33, Hopewell, VA, USA).

2.2. Bacterial strains and growth conditions

Four clinical isolates of *S. aureus* from a repository collected from patients admitted for treatment not related to research at the San Antonio Military Medical Center (Fort Sam Houston, TX, USA) were used in this study [14]. Characteristics of the four clinical isolates used in this study, which were previously confirmed to be positive for biofilm formation, are described in Table 1. UAMS-1 (ATCC strain 49230) is a methicillin-susceptible *S. aureus* strain of the USA200 clonal group and a well-characterized osteomyelitis isolate [29,30]. Xen36 is a bioluminescent strain modified with the *luxABCDE* operon (Caliper Life Sciences Inc.) derived from a methicillin-sensitive clinical bacteremia isolate of *S. aureus* subsp. Wright (ATCC 49525). All bacterial strains were cultured in tryptic soy broth (TSB) with agitation or on blood agar plates overnight at 37 °C.

2.3. Biofilm formation and dispersal assays

Biofilm formation was assessed under static conditions using polystyrene 96-well plates (Corning, Inc., Corning, NY, USA) as described previously [31,32]. Briefly, overnight bacterial cultures were diluted to an OD_{600} of 0.1 in TSB ($\sim 10^7$ CFU/mL), and 20 μL were added to individual wells filled with 180 μl of media and incubated at 37 °C for 48 h. To assess the biofilm dispersal activity of D-AAs, the culture medium from biofilms was removed after 48 h and 200 μL fresh medium containing either an individual D-AA or an equimolar mixture of D-AAs (1:1:1: D-Met:D-Pro:D-Trp) were added at the indicated concentrations. We chose this particular combination because of the individual D-AAs broad activity in the in vitro evaluation against clinical isolates. After treatment with D-AA(s) for 24 h, plates were gently washed with 1× phosphate buffered saline (PBS) to remove unattached cells, stained with 0.1% (w/v) crystal violet (Sigma Aldrich, St. Louis, MI, USA) for 10 min, rinsed with PBS, and then solubilized with 80% (v/v) ethanol. Biofilm biomass was determined by measuring the absorbance of solubilized stain at 570 nm using a microtiter plate reader. For assays measuring the ability of D-AA to block biofilm formation, cells were grown under biofilm conditions as above in the presence of media containing D-AAs. Representative images of the plates of CV-stained biofilms following treatment with D-AA prior to solubilization were taken using a digital camera. All assays were repeated in triplicate with a minimum of four technical replicates.

2.4. Cell viability assays

Human dermal fibroblasts and osteoblasts (PromoCell, Heidelberg, Germany) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Grand Island, NY, USA) supplemented with 10% Fetal Bovine Serum (FBS) and 1× penicillin/streptomycin at 37 °C in 5% CO_2. Prior to each assay, cells were seeded at 100% confluence in black-clear bottom 96-well plates. After 24 h cells were exposed to media containing D-AA (1 nM-50 mM) and incubated for 24 h. Following treatment, cells were washed, re-suspended in 100 μL of sterile saline, and assessed for viability using the CellTiter-Fluor Cell Viability Assay (Promega, Madison, WI, USA) following the manufacturer's instructions. Viability assays were performed in triplicate with a minimum of four replicates. Viability was reported as the percentage of viable cells relative to untreated controls.

2.5. Synthesis of polyurethane (PUR) scaffolds

Polyester triols with a molecular weight of 900 g mol^{-1} and a backbone comprising 60 wt% ε-caprolactone, 30% glycolide, and 10% lactide (T6C3G1L900) were synthesized using published techniques [33,34]. Appropriate amounts of dried glycerol and ε-caprolactone, glycolide, DL-lactide, and stannous octoate (0.1 wt-%) were mixed in a 100-ml flask and heated under an argon atmosphere with mechanical stirring to 140 °C for 24 h. The polyester triol was subsequently washed with hexane and dried. The appropriate amounts of each D-AA (as received from the vendor) were pre-mixed. Next, the polyester triol, LTI-PEG prepolymer (excess isocyanate 15%), 2.0 parts per hundred parts polyol (pphp) tertiary catalyst, 3.0 pphp water, 4.0 pphp calcium stearate pore opener, and the equimolar mixture of D-AAs (0–10 wt% total D-AA, 1:1:1 mixture of D-Met:D-Pro:D-Trp) were loaded into a 20 ml cup and mixed for 1 min using a Hauschild SpeedMixer DAC 150 FVZ-K vortex mixer (FlackTek). The reactive mixture was allowed to cure and foam at room temperature for 24 h. Cylindrical samples for *in vivo* testing (3 mm diameter × 6.5 mm height) were cut using a coring tool and then sterilized by treating with ethylene oxide (EO).

2.6. Characterization of PUR scaffolds

Scaffold density was determined from mass and volume measurements of cured samples, from which the gravimetric porosity was calculated as the volume fraction of pores as described previously [35]. After curing, PUR sections were sputter-coated with gold and imaged using a Hitachi 4200 SEM. Pore size was determined from the SEM images using MetaMorph 7.1 Image Analysis software (MDS Analytical Technologies). Compressive mechanical properties of the scaffolds were measured using a TA Instruments Q800 Dynamic Mechanical Analyzer (DMA, New Castle, DE). Samples were tested after 24 h or 7 days of incubation in PBS. Stress–strain curves were generated by compressing wet cylindrical 6 mm × 6 mm samples at 37 °C at a rate of 0.1 N/min until they reached 60% strain. The compressive modulus was determined from the slope of the initial linear region of each stress–strain curve. Since the scaffolds could not be compressed to failure due to their elasticity, the compressive stress was reported at 50% strain [36].

2.7. Scanning electron microscopy (SEM) analysis of biofilm formation

SEM analysis was performed to examine the effect of augmentation with D-AAs on bacterial attachment and biofilm formation on the scaffold *in vitro* and *in vivo*. PUR scaffolds were fixed with 2% (w/v) glutaraldehyde, 2% (w/v) paraformaldehyde (PFA), 0.15 M sodium cacodylate, 0.15% (w/v) alcian blue for 3 h, rinsed 3× with 0.15 M sodium cacodylate buffer, and incubated in 1% (v/v) osmium tetroxide in sodium

Table 1
Description of *S. aureus* strains investigated in this study.

Strain	Strain characteristics	Biofilm formation[a]
UAMS-1	ATCC strain 49230. Methicillin-susceptible strain of the USA200 clonal group and a well-characterized osteomyelitis isolate	Strong
Xen36	Xen36 is a bioluminescent strain modified with the *luxABCDE* operon derived from a methicillin-sensitive clinical bacteremia isolate of *S. aureus* subsp. Wright (ATCC 49525)	Weak
S. aureus Clinical Isolate 1	Methicillin-resistant strain of the USA300 clonal group; wound isolate	Strong
S. aureus Clinical Isolate 2	Methicillin-resistant strain of the USA300 clonal group; blood isolate	Weak
S. aureus Clinical Isolate 3	Methicillin-resistant strain of the USA700 clonal group; cultured from deep would	Strong
S. aureus Clinical Isolate 4	Methicillin-resistant strain of the USA200 clonal group; cultured from deep wound	Strong

[a] Biofilm formation classification is based on previous studies comparing the biofilm forming capacity compared to a biofilm positive control, *S. epidermidis* ATCC 12228; strong indicates biofilm $\geq$ than *S. epidermidis* and weak biofilm former $\leq$ than the control as determined by microtiter plate assay [14].

cacodylate for 1 h. Samples were dehydrated with a stepwise gradient of ethanol and then treated with hexamethyldisilizane prior to drying in a desiccator overnight. Samples were sputter-coated with gold palladium and viewed with a Hitachi 4200 or JEOL-6610 scanning electron microscope.

2.8. D-AA release kinetics

PUR scaffolds incorporating 10 wt% of a 1:1:1 mixture of D-Met:D-Pro:D-Trp were incubated in PBS for up to 8 weeks. The medium was sampled twice weekly and analyzed for D-AAs by HPLC using a system equipped with a Waters 1525 binary pump and a 2487 Dual-Absorbance Detector at 200 nm. Samples of released D-AAs were eluted through an Atlantis HILIC Silica column (5 μm particle size, 4.6 mm diameter x 250 mm length) using an isocratic mobile phase flowing at 1 mL/min [37]. The mobile phase contained 2.5 mM potassium dihydrogen phosphate with pH = 2.85 (A) and Acetonitrile (B) at a ratio of A25:B75. The column oven temperature was maintained at 30 °C. Sample concentration was determined in reference to an external standard curve using the Waters Breeze system. Standard curves were prepared in the following concentration ranges: (1) 7.8 μg/mL to 1 mg/mL for D-Met and D-Pro and (2) 0.78 μg/mL to 100 μg/mL for D-Trp.

2.9. Bacterial adhesion to PUR scaffolds

Bacterial adherence and biofilm formation on scaffolds with or without D-AAs was evaluated as described previously [38]. The study design is listed in Table 2. Sterile blank PUR scaffolds with no D-AAs were utilized as a negative control (PUR (−)). Blank scaffolds (denoted as PUR) or scaffolds augmented with an equimolar mixture of D-AAs (denoted as PUR + D-AA-x, where $x = 0.1, 1.0, 5.0$, or 10 wt% 1:1:1 mixture of D-Met:D-Pro:D-Trp) were placed into 24-well polystyrene plates containing sterile PBS for 2 h at room temperature, which allowed the scaffolds to become saturated. Samples were then transferred into a bacterial suspension of UAMS-1 (10^7 CFU/mL) in PBS and exposed for an additional 2 h at 37 °C with agitation in 24-well plates. Following exposure, scaffolds were rinsed with PBS to remove non-attached bacteria and incubated overnight in TSB at 37 °C to allow adequate time for attached bacteria to develop biofilms. Following incubation, scaffolds were then placed in 1 mL PBS and sonicated for 10 min using a low-power bath sonicator. Bacterial CFUs per volume of scaffold were determined by plating serial dilutions on blood agar plates. Bacterial attachment and biofilm formation on scaffolds following incubation were also evaluated by SEM analysis. The sample size was 3, which were performed in duplicate.

Table 2
Study design investigating the ability of biofilm-dispersive scaffolds to reduce bacterial contamination *in vitro*. Scaffolds were contaminated with 10^7 CFU/ml *S. aureus* for 2 h and outcomes were assessed after 24 h incubation time in PBS ($n = 4$).

Group	Description	24 h
PUR (−)	Sterile blank PUR scaffold with no D-AAs	4
PUR	Contaminated blank PUR scaffold	4
PUR + D-AA-0.1	Contaminated blank PUR scaffold augmented with 0.1% D-AAs	4
PUR + D-AA-1	Contaminated blank PUR scaffold augmented with 1.0% D-AAs	4
PUR + D-AA-5	Contaminated blank PUR scaffold augmented with 5.0% D-AAs	4
PUR + D-AA-10	Contaminated blank PUR scaffold augmented with 10% D-AAs	4

2.10. Rat femoral 6-mm segmental defect model

This study was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals. A previously characterized contaminated critical size defect in rat (Sprague–Dawley; 373 ± 4.15 g) femurs was utilized as the *in vivo* model of infection [39–41]. The study design is listed in Table 3. Briefly, a 6-mm segmental defect was created using a small reciprocating saw blade (MicroAire 1025, MicroAire, Charlottesville, VA), stabilized with a polyacetyl plate (length 25 mm, width 4 mm and height 4 mm) and fixed to the surface of the femur using threaded K-wires. Blank PUR scaffolds implanted in a sterile defect were utilized as a negative control (PUR (−)) and for SEM analysis to distinguish between host cellular and bacterial infiltration of the scaffolds. The defects in all other animals were then implanted with 30 mg of type I bovine collagen (Stryker Biotech, Hopkinton, MA, USA) wetted with 10^2 CFU of *S. aureus* strain Xen36 (Caliper Life Science, Hopkinton, MA) or *S. aureus* strain UAMS-1. The Xen36 strain is a weak biofilm producer and was used as a negative control. Six hours after contamination, the wounds were opened, debrided, and irrigated with saline. PUR or PUR + D-AA-x (1.0, 5.0, or 10 wt% 1:1:1 mixture of D-Met:D-Pro:D-Trp) scaffolds were then implanted into the wounds. Since cefazolin is recommended for primary prevention of infections associated with open fractures [42], rats received systemic antimicrobial treatment with cefazolin (5 mg/kg) administered subcutaneously twice a day for 3 days post surgery. Two weeks following surgery, the rats were euthanized and the femurs were weighed, snap-frozen in liquid nitrogen, ground to a fine powder, and re-suspended in saline. CFUs (expressed as $\log_{10}$ CFU/g tissue) were determined by plating serial dilutions onto blood agar plates and incubated at 37 °C for 24 h. Scaffolds from PUR (−), PUR, and PUR + D-AA-10 groups were evaluated by SEM.

2.11. Statistical analysis

For *in vitro* comparisons of groups, statistical analyses were performed using a One-Way ANOVA with a Bonferroni test to determine statistical differences between groups. Non-parametric statistical methods were used to analyze the results from the *in vivo* study. Contingency tables analyzed with a Fisher's exact test were used to compare the number of infected and non-infected samples between groups. The CFU counts of the different treatment groups were compared using the Kruskal–Wallis test followed by a Dunn's multiple comparison test to identify differences between groups. Non-parametric analyses were performed using GraphPad InStat Version 3.0 (GraphPad software, San Diego California, USA). $P < 0.05$ was considered statistically significant.

Table 3
In vivo study design investigating the ability of biofilm-dispersive scaffolds to reduce contamination in 6-mm segmental defect in rat femora contaminated with 10^2 CFU *S. aureus* UAMS-1 or Xen36. Outcomes were assessed at 2 weeks ($n = 10$).

Group	Description	No infection	UAMS-1	XEN36
PUR (−)	Blank PUR scaffold in a sterile defect	10	0	0
Empty	Contaminated defect not grafted with a scaffold	0	10	10
PUR	Blank PUR scaffold in a contaminated defect	0	10	10
PUR + D-AA-1	PUR scaffold with 1.0% D-AAs in a contaminated defect	0	10	0
PUR + D-AA-5	PUR scaffold with 5.0% D-AAs in a contaminated defect	0	10	0
PUR + D-AA-10	PUR scaffold with 10% D-AAs in a contaminated defect	0	10	10

3. Results

3.1. D-amino acids activity in vitro

To initially evaluate the feasibility of local delivery of D-AAs as a biofilm-dispersive scaffold, the activity of D-AAs on biofilm dispersal and prevention was tested *in vitro* on a panel of four clinical isolates (Table 1) of *S. aureus*. Pre-screening of eight individual D-AAs identified four amino acids, including D-Met, D-Phe, D-Pro, and D-Trp, as highly effective at dispersing biofilms formed by the four clinical isolates (Fig. 1), whereas the other four D-AAs had minimal effects. D-AAs dispersed biofilms in a dose-responsive manner and were most effective at concentrations $\geq$5 mM. Thus, 5 mM was chosen as the concentration for future studies. The efficacy of D-AAs varied between different bacterial strains, although for each strain tested more than one of the four D-AAs was effective at dispersing biofilms. Consistent with previous studies, the anti-biofilm effect was isomer-specific, as no dispersal activity was observed with L-isomers of D-AAs (data not shown). When tested against the panel of clinical isolates of methicillin-resistant *S. aureus* ($n = 5$), D-Phe, D-Met, D-Trp, and D-Pro were effective at dispersing established biofilms *in vitro* as determined by the measurement of the biofilm biomass (Fig. 2A, C). In addition to dispersing established biofilms, the four identified D-AAs also significantly blocked formation of biofilms by the clinical strains when bacteria were cultured in the presence of D-AAs (Fig. 2B). When combined as an equimolar mixture of D-Met, D-Pro, and D-Trp, biofilm-dispersive activity was enhanced (Fig. 2D–E), as suggested by the decrease in biofilm biomass observed at D-AA concentrations 1 mM (which was not observed for the individual D-AAs). Importantly, D-AAs had no significant effect on the growth of the bacteria, indicating that biofilm dispersal was a specific property and not the result of growth inhibition (Supplemental Fig. 1).

3.2. Cytotoxicity of D-AAs in vitro

While Figs. 1 and 2 show that D-AAs both block biofilm formation as well as disperse established biofilms, the toxicity of D-AAs toward mammalian cells has not been extensively investigated. Thus, we evaluated the cytotoxicity of D-AAs *in vitro* using human osteoblasts and dermal fibroblasts that are relevant to bone and wound healing, respectively. Osteoblasts and fibroblasts exposed to up to 50 mM of D-Met, D-Phe, D-Pro showed >70% viability after 24 h. Cytotoxicity was observed in mammalian cells exposed to D-Trp at

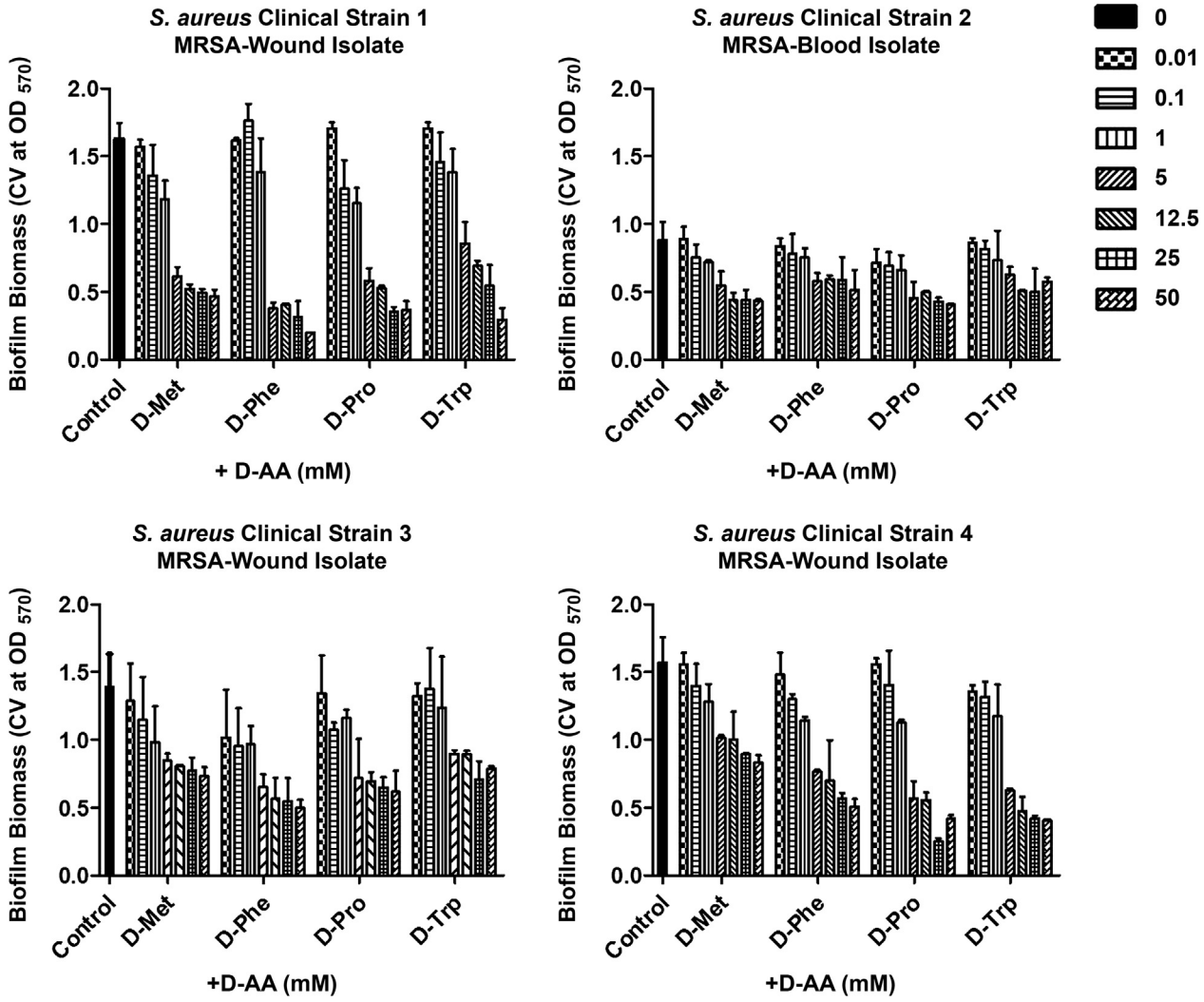

Fig. 1. Screening of D-amino acids against clinical strains of *S. aureus*. Screening of D-Met, D-Phe, D-Pro, and D-Trp at concentrations ranging from 0.001 mM to 50 mM against preformed biofilms of four representative clinical isolates of *S. aureus* (described in Table 1). Biofilm dispersal was assessed by quantitating the remaining biofilm biomass following treatment with D-AAs by measuring the absorbance of solubilized CV from the stained biofilms at 570 nm.

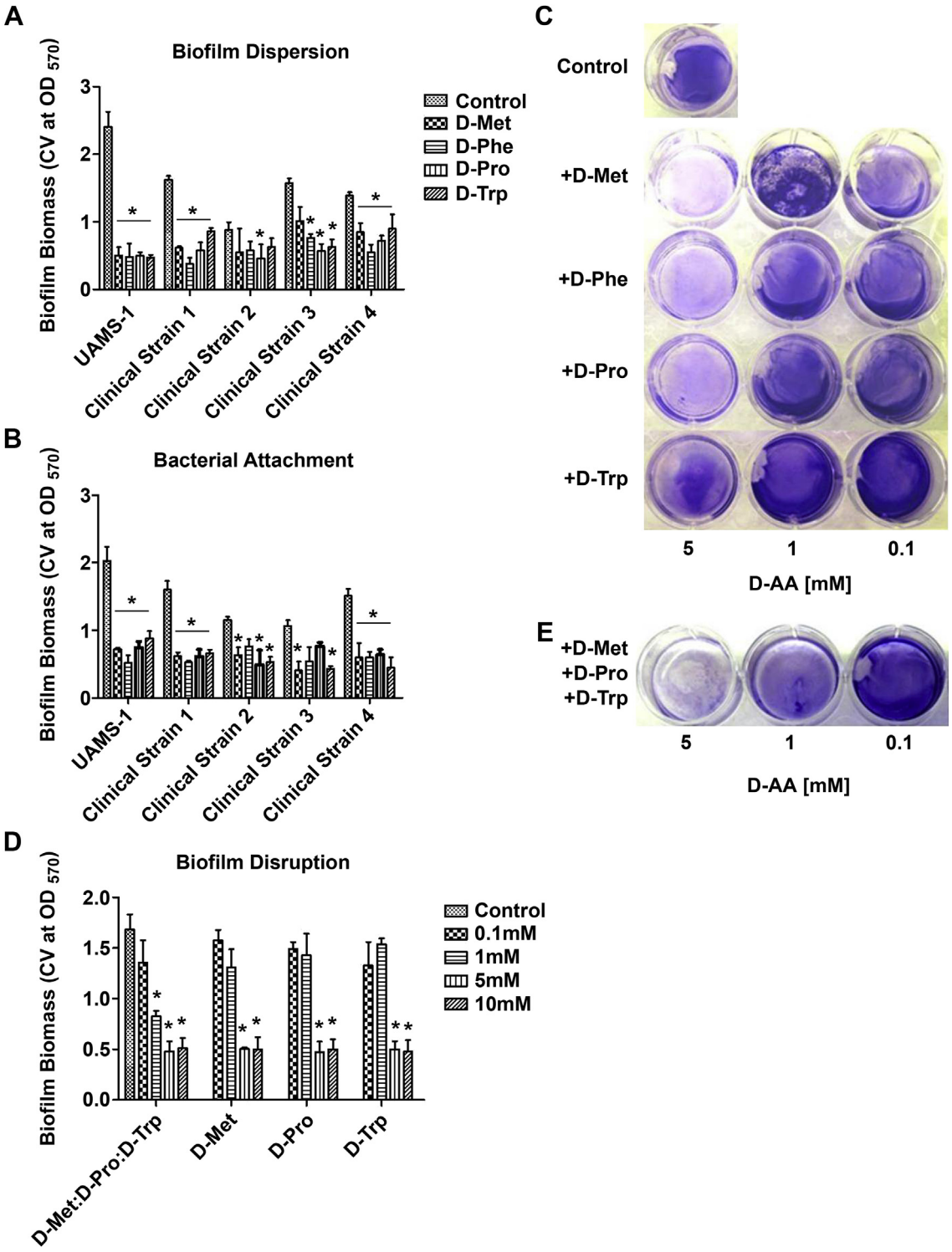

Fig. 2. D-amino acids disperse biofilms and prevent biofilm formation in clinical isolates of *S. aureus*. (A) Dispersion of pre-formed biofilms: Biofilm biomass (OD_{570}) following treatment of pre-formed biofilms of four representative clinical isolates of *S. aureus* (described in Table 1) with 5 mM of each individual D-AA for 24 h at 37 °C. (B) Prevention of biofilm formation: Biofilm biomass for the same clinical isolates as above following co-incubation of the bacteria with 5 mM of D-AA. (C) Representative images of CV-stained biofilms from *S. aureus* UAMS-1 (bone isolate) following overnight treatment with individual D-AAs. (D) An equimolar mixture ofD-AAs is more effective at dispersing biofilms than individual **D**-AAs. Biofilm biomass (OD_{570}) following treatment of pre-formed biofilms of *S. aureus* UAMS-1 with an equimolar mixture (0.1 − 5 mM total concentration) of D-Met, D-Pro, and D-Trp for 24 h at 37 °C. **(E)** Representative images of CV-stained biofilms from *S. aureus* UAMS-1 following overnight treatment with the mixture of D-AAs (0.1 − 5 mM). Averages are representative of three independent experiments, error bars signify standard deviation. Statistical analysis was performed using a One-Way ANOVA followed by a Bonferroni test to identify differences between groups; $p < 0.05$ was considered to be statistically different from the control group.

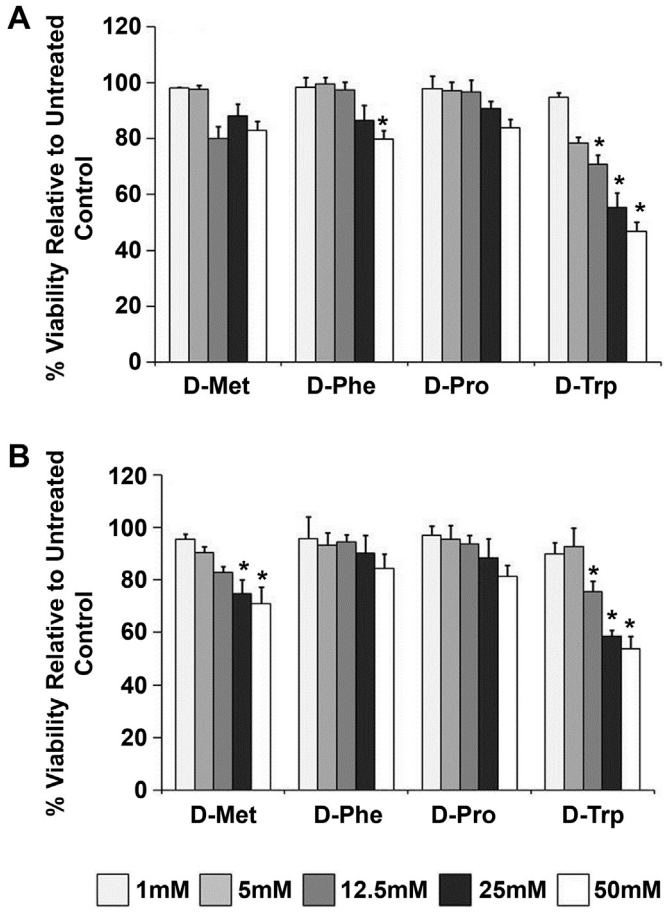

Fig. 3. D-amino acids have limited cytotoxicity *in vitro*. Viability of human osteoblasts (A) and dermal fibroblasts (B) exposed to media supplemented with D-Met, D-Phe, D-Pro, and D-Trp (1–50 mM) for 24 h at 37 °C in 5% CO_2. Cell viability was determined using the Cell-Titer Flour assay by measuring fluorescence $405_{ex}/505_{em}$ and is represented as a percentage viability relative to non-treated controls. Values represent the average of three independent experiments, and error bars indicate standard deviation. Statistical analysis was performed using a One-Way ANOVA followed by a Bonferroni test to identify differences between groups; $p < 0.05$ was considered to be statistically different from controls (*).

concentrations exceeding 12.5 mM (~60% viability) (Fig. 3A–B). Importantly, these studies indicate that the D-AAs have minimal cytotoxic effects on mammalian cells at or above concentrations observed to be effective for preventing and disrupting biofilms *in vitro*.

3.3. Scaffold characteristics and in vitro release

Previous experiments investigating the feasibility of biofilm dispersion by exogenous D-AAs have focused on 2D surfaces. As an initial step toward the creation of a biofilm-dispersive scaffold, two-component PUR scaffolds prepared by reactive liquid molding were augmented with a mixture of D-AAs (1:1:1 wt% D-Met:D-Pro:D-Trp) as a labile powder. Prior to *in vivo* testing, the PUR + D-AA scaffolds were characterized *in vitro*. Scaffolds containing 0 (PUR) or 10 wt% D-AA mixture (PUR + D-AA-10) had similar values of density, porosity, and pore size before and after leaching overnight in PBS. Representative SEM images of the PUR and PUR + D-AA-10 scaffolds show inter-connected pores and a mean pore diameter ranging from 370 to 378 μm (Fig. 4A). While the addition of 10% D-AA mix to the PUR scaffolds did not affect the porosity, the wet mechanical properties were significantly reduced compared to the empty scaffold (Fig. 4B). There were no differences in the properties of the scaffolds incubated in PBS for 24 h or 7 days.

The release kinetics of D-Pro, D-Met, and D-Trp were characterized by an initial burst followed by a sustained release for up to 21 days (Fig. 4C). D-Met released the fastest, characterized by a 60% burst on day 1 and nearly 100% release by day 14. The release of D-Pro was somewhat slower (45% burst and 85% release by day 28), while D-Trp released slowly, with only a 25% burst and 44% release after 28 days. The Weibull equation has been used to identify the mechanism controlling drug release from polymeric materials [39,43]:

$$M_t/M_\infty = 1 - \exp\left(-at^b\right)$$

where M_t corresponds to the mass of drug released in time t, M_∞ is the mass of drug released at infinite time (which corresponds to the initial loading of drug), and a and b are constants. When $b < 0.75$, Fickian diffusion controls drug release, while a more complex mechanism involving both diffusion and swelling controls release when $b > 0.75$ [44]. The D-AA release data were fit to the Weibull model and the values of the b parameter for D-Met, D-Pro, and D-Trp were calculated as 0.56, 0.35, and 0.21 respectively, suggesting that the release of each D-AA from the scaffolds was diffusion-controlled.

3.4. Effects of biofilm-dispersive scaffolds in vitro

The effect of local delivery of D-AAs from biofilm-dispersive scaffolds on bacterial contamination was evaluated *in vitro* prior to *in vivo* testing. Incorporation of D-AA into PUR scaffolds at concentrations ≥1 wt% D-AA significantly reduced the amount of attached bacteria and biofilm formation on the surface compared to the scaffolds without D-AA. PUR scaffolds with 1, 5, and 10 wt% D-AA had a ≥ 4-log reduction in the number of bacteria (Fig. 5A), while PUR scaffolds with 0.1% showed a more moderate (~1-log reduction) but significant reduction in bacteria attached to the scaffold surface. Consistent with the bacterial counts, SEM images of PUR scaffolds augmented with D-AA also demonstrated the dramatic reduction in surface-attached bacteria within biofilms on scaffolds augmented with the D-AA mixture (Fig. 5B). However, as indicated by the bacterial counts, PUR scaffolds without D-AA as well PUR scaffolds with 0.1% D-AA had extensive bacterial colonization and the presence of biofilms on the surface.

3.5. Effects of biofilm-dispersive scaffolds in vivo

For the *in vivo* studies, 6-mm segmental defects in rats were contaminated with 10^2 CFU *S. aureus* Xen36, a bioluminescent, septicemic isolate forming weak biofilms, or 10^2 CFU *S. aureus* UAMS-1, an osteomyelitis isolate and a strong biofilm producer. Treatment of femoral UAMS-1-contaminated defects with PUR + D-AA-5 or PUR + D-AA-10 significantly reduced bacterial contamination within the homogenized bone ($p < 0.05$) (Fig. 6A), while lower doses did not reduce contamination compared to the empty (untreated) defect control. Similarly, PUR + D-AA-5 and PUR + D-AA-10 reduced the number of contaminated samples compared to the PUR scaffold (Fig. 6B), although the difference did not reach statistical significance ($p = 0.087$). Consistent with these observations, SEM analysis of scaffolds removed from rats following infection also showed a dramatic reduction of biofilm attached to the surface of the scaffolds (Fig. 7). Blank PUR scaffolds implanted in contaminated defects exhibited extensive bacterial adhesion and biofilm formation on the majority of the surface, whereas PUR + D-AA-10 showed a substantial reduction in the amount of attached

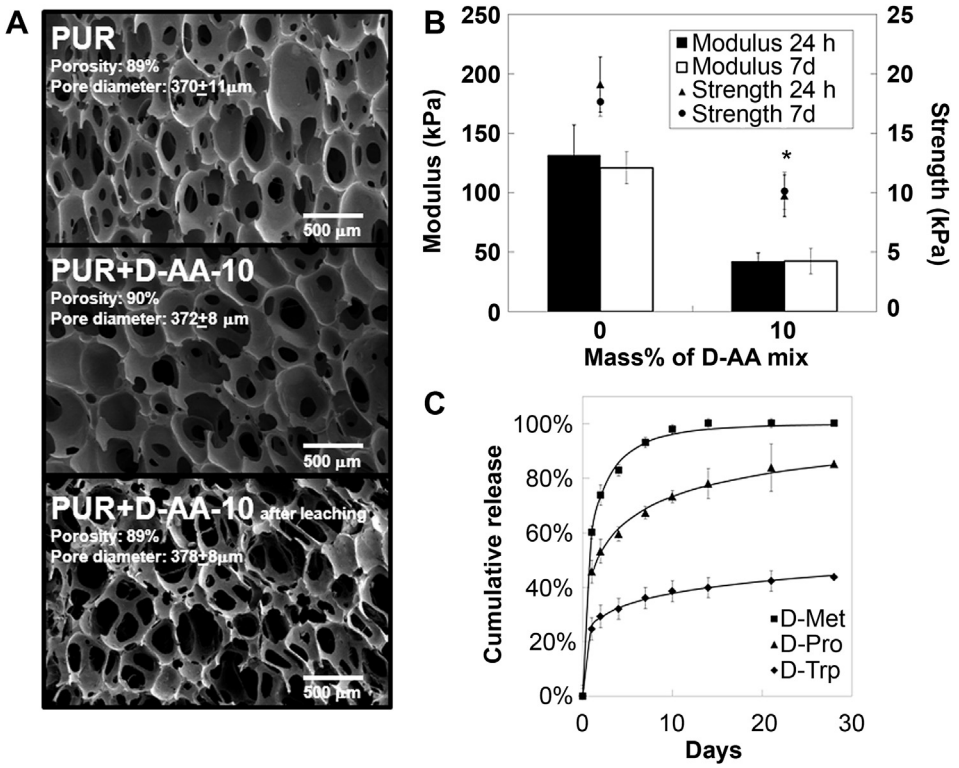

Fig. 4. Characterization of PUR + D-AA scaffolds. (A) SEM images of PUR, PUR + D-AA-10 (before leaching), and PUR + D-AA-10 (after 24 h leaching) scaffolds. Porosity and pore size remain relatively constant with increasing wt% D-AA. (B) Compressive mechanical properties of dry and wet (soaked in PBS for 24 h) PUR and PUR + D-AA-10 samples; $p < 0.05$ was considered to be statistically different from controls (*). (C) Cumulative % release of D-Pro, D-Met, and D-Trp versus time (symbols). The solid lines represent the fit to the Weibull model.

bacteria. In contrast, PUR + D-AA scaffolds implanted in defects contaminated with 10^2 CFU Xen36 strain, an extremely weak biofilm producer, did not significantly reduce bacterial contamination or the number of contaminated samples compared to the empty defect (Supplemental Fig. 2).

4. Discussion

Despite meticulous clinical management including surgical debridement and the use of systemic antibiotics, contamination rates of open fractures continue to be a significant cause of non-osseous union, potentially leading to extremity amputation. Local delivery of antibiotics from bone grafts has been investigated as a strategy to reduce bacterial contamination and promote osseous union [39,45–52]. However, the efficacy of antibiotics against the surface-attached communities of bacteria known as biofilms, which are considered a major virulence factor in chronic disease [4,6], is limited due to the slow metabolic and growth rates of bacteria within the biofilm [53]. Furthermore, the avascular bone graft may itself serve as a substrate for bacterial colonization and a nidus for recurrent infections [54]. Finally, a small population (i.e., 0.1–10%) of "persister cells" survives antimicrobial therapy and rapidly grows after the cessation of antibiotic therapy, potentially resulting in recurrent infections [55]. Recent studies have

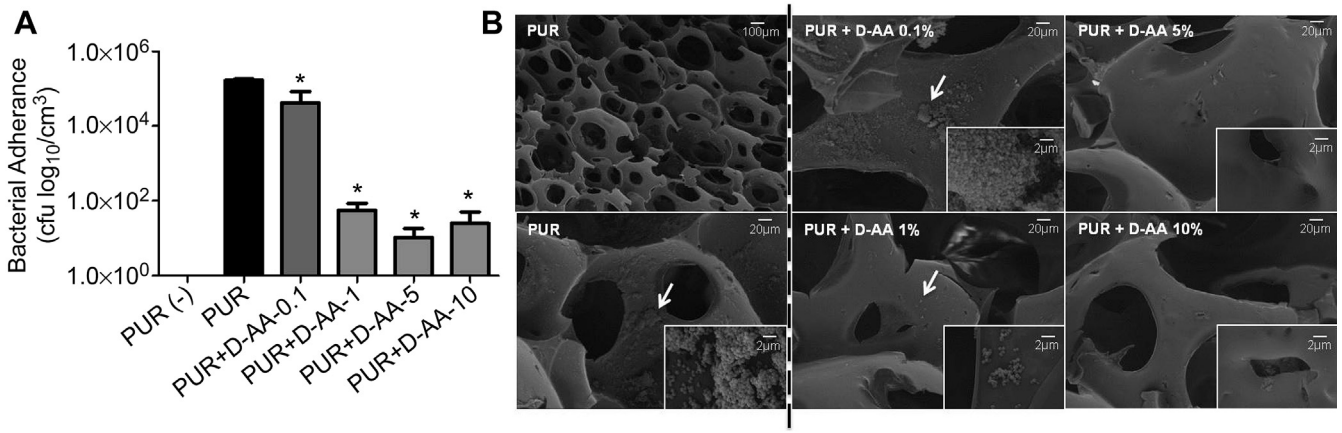

Fig. 5. Augmentation of PUR scaffolds with an equimolar mixture ofD-AAs reduces bacterial adherence *in vitro*. (A) $\log_{10}$ CFU/cm^3 UAMS-1 bacteria adhered to PUR scaffolds augmented with D-AAs after 24 h incubation time decrease ~4 orders of magnitude for ≥1 wt% equimolar D-AA mixture. The PUR negative control (PUR (−)) incubated in sterile medium shows no contamination. (B) SEM images of PUR + D-AA scaffolds exhibiting decreased biofilm with increasing D-AA concentration.

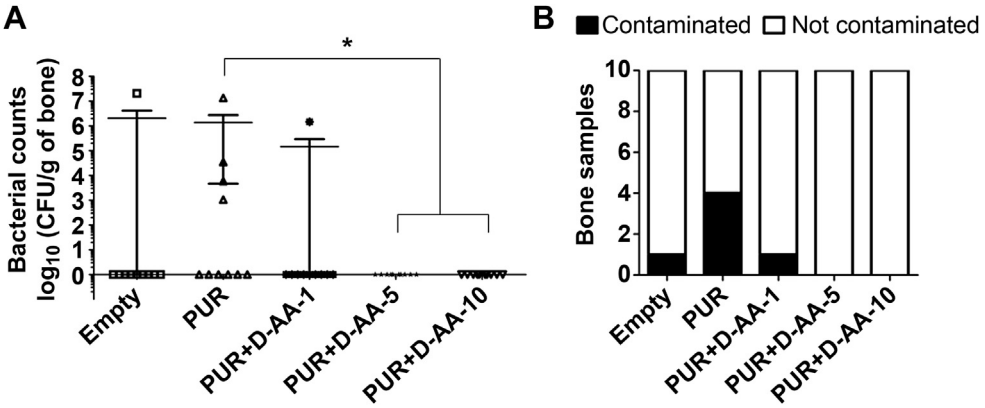

Fig. 6. Augmentation of PUR scaffolds with an equimolar mixture of D-AAs reduces bacterial contamination of segmental defects contaminated with 10^2 CFU *S. aureus* UAMS-1 *in vivo*. (A) Bacterial counts ($\log_{10}$ CFU/g) in homogenized bone from segmental defects of rats contaminated with 10^2 CFU of *S. aureus* UAMS-1 followed by implantation of no scaffold (Empty, $n = 10$), PUR blank scaffold (PUR, $n = 10$), or PUR scaffold + equimolar D-AA mixture ($n = 10$ per group) for two weeks post-wounding. Bars represent the mean value and error bars are the standard error of the mean. Statistical analysis was performed using a Kruskal–Wallis test followed by a Dunn's multiple comparisons test to identify differences between groups (* significantly different than PUR, $p < 0.05$) (B) Distribution of contaminated and non-contaminated bone samples from the segmental defects. Fewer samples were contaminated when the PUR scaffold was augmented with D-AA content ≥5 wt%, although the differences were not statistically significant ($p = 0.087$). Statistical analysis was performed using contingency tables analyzed with a Fisher exact test comparing the number of contaminated bone samples for each PUR + D-AA treatment group to the PUR blank scaffold.

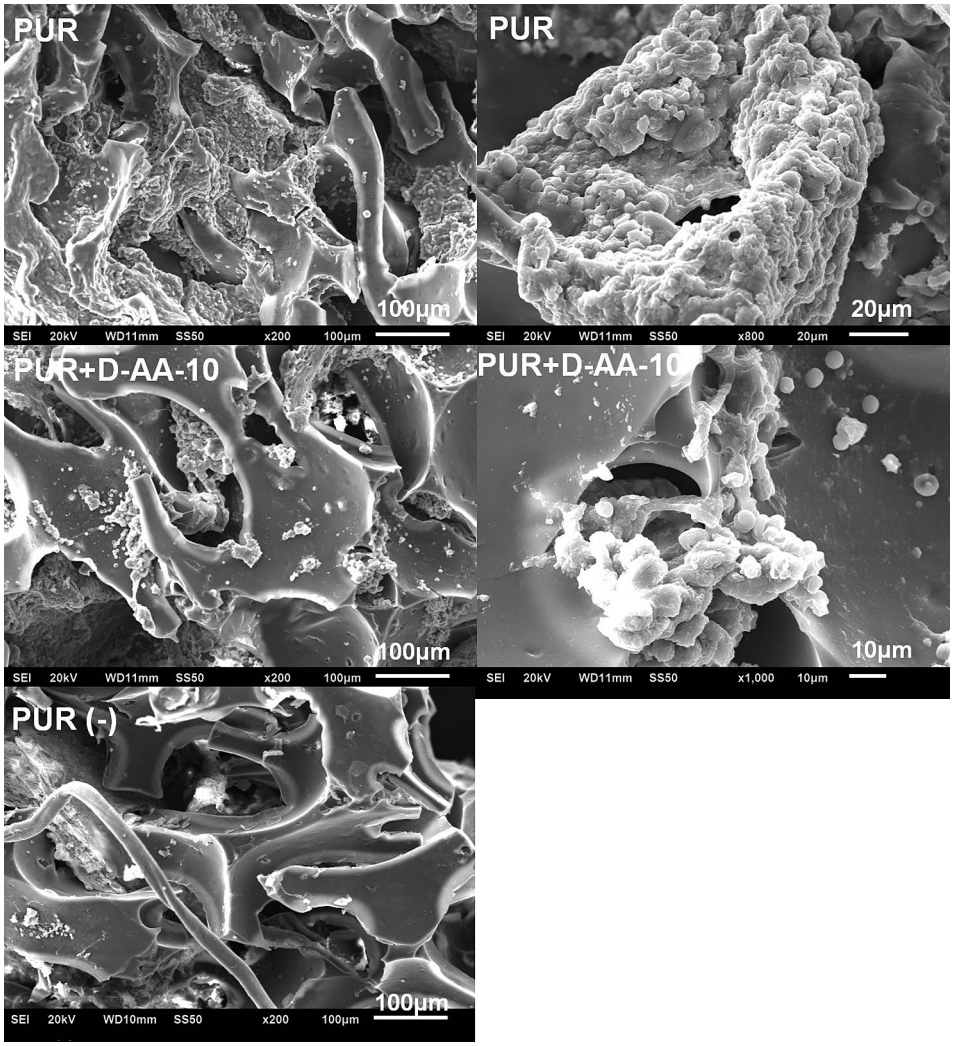

Fig. 7. Low- and high-magnification SEM images of biofilms on PUR and PUR + D-AA-10 scaffolds implanted in contaminated femoral segmental defects in rats for 2 weeks show reduced bacterial adhesion for the scaffold augmented with 10 wt% of the equimolar mixture of D-AAs. PUR scaffolds implanted in sterile defects (PUR (−) negative control) show minimal bacterial adhesion.

highlighted the potential of bacterial signaling molecules that trigger biofilm dispersal, such as bismuth thiols [20,56], quorum-sensing inhibitors and analogs [57], and D-AAs [26,27], as therapeutic agents for treatment of chronic infections. In this study, we have shown that local delivery of D-AAs from PUR scaffolds inhibits biofilm formation by clinical isolates of *S. aureus* both *in vitro* and *in vivo*. Dose-response experiments showed that D-AAs inhibited biofilm formation and dispersed existing biofilms at concentrations ≥5 mM *in vitro*. A PUR scaffold augmented with ≥5 wt% D-AAs significantly reduced bacterial contamination and biofilm formation by the strong biofilm-producing strain *S. aureus* UAMS-1 compared to the control scaffold with no D-AAs in a contaminated segmental defect in rats. Interestingly, with careful scanning of the scaffolds with D-AAs, small colonies of bacteria within a biofilm could be found. Conversely, biofilm formation could easily be found on the scaffolds without D-AAs that were retrieved from contaminated defects. Almost the entire scaffold was covered with biofilm, and the colonies were very robust. Furthermore, D-AAs exhibited relatively low cytotoxicity to mammalian cells at doses effective at inhibiting biofilm formation.

Considering the many human chronic diseases involving biofilms, the use of biofilm dispersal agents has gained considerable interest for the treatment of biofilm-mediated infections. A previous study has reported that D-Phe, D-Pro, and D-Tyr prevent biofilm formation and trigger biofilm dispersion in *S. aureus* WT strain SC01 at concentrations as low as 0.5 mM *in vitro* [27]. In this study, we have shown that D-Met, D-Phe, D-Pro, and D-Trp inhibit biofilm formation and disperse established biofilms of clinical strains of *S. aureus* at concentrations ≥5 mM (Fig. 1), which is ~10 times greater than the previously reported dose. The activity of individual D-AAs varied in previous studies, with D-Tyr requiring the lowest concentration (3 μM), and D-Met (2 mM), D-Trp (5 mM) and D-Leu (8.5 mM) requiring higher concentrations for anti-biofilm activity against *Bacillus subtilis* strain NCIB 3610 and for *S. aureus* strain SC01 [26]. Differences between bacterial species as well as strain heterogeneity are likely contributing factors to the observed discrepancies between studies. Importantly, from our studies we identified concentrations having effective biofilm-dispersive activity against a number of clinical strains. Consistent with a previous study showing that the equimolar mixture of D-Phe:D-Pro:D-Tyr lowered the effective dose [27], the data in Fig. 2D–E show that an equimolar mixture of D-Met:D-Pro:D-Trp shifted the dose–response curve toward lower doses compared to the individual D-AAs. Other than for *B. subtilis*, the mechanisms by which D-AAs disrupt biofilms are not known. However, the enhanced anti-biofilm activity of the D-AA mixture is suggestive of multiple mechanisms acting on the bacteria, which warrants further investigation.

In order to be useful as a clinical therapy, D-AAs must exhibit minimal cytotoxicity at concentrations that are effective at dispersing biofilms. As shown in Fig. 3, osteoblasts and fibroblasts treated with D-Phe, D-Pro, or D-Met for 24 h exhibited >70% viability at concentrations ≤50 mM, while D-Trp exhibited cytotoxic effects (i.e., <70% viability) at concentrations >12.5 mM. While these data suggest that individual D-AAs are non-cytotoxic to mammalian cells at concentrations efficacious against biofilms, they contrast with a previous study reporting cytotoxicity of D-Phe, D-Met, and D-Trp toward Chinese Hamster Ovary (CHO) and HeLa cells at concentrations ≥10 mM [28]. The discrepancies in D-AA toxicities between the present study and that reported previously may be attributed to differences among cell lines. In another study, D-Phe and D-Trp reportedly elicited a chemotactic response in human neutrophils via activation of GPR109B [58]. Additional *in vivo* studies investigating the biocompatibility of D-AAs in sterile defects are needed to further assess their safety, and these studies are currently ongoing in our laboratories.

As a first step toward the development of a biofilm-dispersive bone graft for clinical applications, we evaluated the effects of local delivery of D-AAs from biodegradable PUR scaffolds on infection in a contaminated segmental defect model in rats. The scaffold investigated in this study has a half-life of 14 weeks *in vitro* [36], and degrades to non-cytotoxic breakdown products such as lysine and α–hydroxy acids [59]. PUR scaffolds are effective delivery vehicles for sustained released of biologics, such as antibiotics [39,60] and recombinant human bone morphogenic protein-2 (rhBMP-2) [40,44,61]. Furthermore, PUR scaffolds are injectable [62,63] and thus can be delivered using minimally invasive surgical techniques. As shown in Fig. 4, while D-AAs had minimal effects on the porosity and pore size, PUR + D-AA scaffolds exhibited approximately 2–3-fold decreases in both Young's modulus and compressive stress at 50% strain. A previous study reported that PUR scaffolds augmented with 8 wt% tobramycin had significantly higher porosity and lower modulus than blank PUR scaffolds after 24 h incubation time in PBS, which was attributed to rapid leaching of tobramycin and consequent formation of new pores [60]. Surprisingly, in the present study the modulus decreased significantly even after 24 h, at which time only a fraction of the D-AAs had released, and did not decrease further for up to 7 days of incubation. These observations suggest that the decrease in modulus with addition of D-AAs results from defects in the pore walls of the scaffold caused by the presence of the particles rather than from the formation of new pores due to leaching of the D-AAs.

As shown in Fig. 4C, PUR + D-AA scaffolds supported diffusion-controlled sustained release of the biologically active drug for up to 4 weeks, which is consistent with previous studies reporting diffusion-controlled release of active antibiotics [39,60], recombinant human growth factors [44,64], and siRNA nanoparticles [65]. At each time point, the order of cumulative release was D-Met > D-Pro > D-Trp, while the order of solubility was D-Pro >> D-Met > D-Trp [66]. At 4 weeks, >85% of D-Pro and D-Met had been released and <10% of the scaffold had degraded [36], which is consistent with the notion that D-AA release was diffusion-controlled at early time points. However, <40% of the D-Trp had been released by 4 weeks, suggesting that degradation of the scaffold may control D-Trp release kinetics at later (>4 weeks) time points. Since the free base form of each D-AA was used in this study, the release kinetics could be increased by using the more soluble hydrochloride as reported previously for vancomycin Ref. [39]. While antibiotic therapy for up to 8 weeks is recommended for effective treatment of MRSA osteomyelitis [67], the optimal release profile for D-AAs is unknown and thus merits further investigation.

In a proof-of-concept study, we evaluated the ability of biofilm-dispersive PUR scaffolds augmented with D-AAs to prevent biofilm formation and reduce CFUs in a contaminated rat segmental defect model. Several recent studies have evaluated the effects of local delivery of antibiotics on infection using an acute contamination model, in which the bone graft was placed immediately after contamination of the defect with bacteria [68,69]. However, preclinical models with an established chronic infection [40,70] represent a more rigorous test for the efficacy of biofilm-dispersive grafts, since they ensure that the bacteria are able to adhere to the surface of the wound and form biofilms [71]. In the present study, rat segmental defects were contaminated with 10^2 CFU for 6 h prior to implantation of the PUR + D-AA scaffolds. Augmentation of the scaffolds with ≥5 wt% D-AAs significantly reduced bacterial contamination within the segmental defects treated with UAMS-1, an osteomyelitis strain. In contrast, the D-AAs had no significant effect on defects contaminated with Xen36, which is a weak biofilm producer [14]. These observations are consistent with the notion that D-AAs reduce contamination in the defect by preventing the formation of and/or dispersing biofilms,

thus limiting the application of D-AAs to treatment of biofilm infections. However, numerous studies indicate that the majority of staphylococcal clinical isolates *in vitro* as well as those found in human tissues are strong biofilm producers.

Because D-AAs are not bactericidal, they are anticipated to be most effective as an adjuvant therapy to conventional treatment with systemic antibiotics. In this study, rats were treated with cefazolin (5 mg/kg) administered subcutaneously for 3-days post surgery to simulate the clinical scenario [42]. Despite the presence of systemic antibiotics, one of the untreated rats developed an infection, which increased to four rats when treated with blank PUR scaffolds suggesting that placement of an avascular graft into an open fracture potentiates infection. Treatment with PUR + D-AA-5 or PUR + D-AA-10 scaffolds reduced the number of infected rats to zero, which underscores the potential clinical utility of biofilm-dispersive bone grafts as an adjuvant therapy to systemic antibiotics. The broad spectrum of D-AAs will allow their use for preventing infection without the need of knowing contaminating bacteria. For chronic infections such as MRSA osteomyelitis, treatment with systemic antibiotics is recommended for a minimum of 8 weeks [67]. Importantly, for most patients, the extended duration of systemic treatment is associated with a number of risks to the patient's health, including renal toxicity, and is an economic burden to the patient and healthcare system. Our results suggest that the local delivery of a biofilm dispersion agent alone or co-delivered with antimicrobial agents represents a potentially efficacious therapy for treatment of chronic infections that when combined with the standard systemic antimicrobial treatment may reduce the time of treatment and resulting complications and cost for chronic orthopedic infections.

5. Conclusions

The ability of bacteria to establish biofilms substantially hinders the treatment of orthopedic infections and is implicated as significant contributing factor in the sequelae associated with open fractures. Biofilm-dispersive scaffolds augmented with D-AAs can be used as a therapeutic strategy to reduce microbial burden within wounds and improve healing outcomes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.06.026.

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